REMARKS

Applicants wish to thank Examiner Xu for the attention accorded to the instant application, and respectfully requests reconsideration of the application as amended.

Claims 1-18 are pending in the present application.

Applicants would like to thank Examiner Xu for speaking with Applicants' representative on April 14, 2010. During the interview the references cited in the non-final rejection dated November 17, 2009 were discussed. Examiner Xu and Applicants' representative came to an agreement that the subject matter of Claim 7 contained patentable subject matter over the cited art. No agreement was reached regarding the other pending claims.

The allowable subject matter of Claim 7 has been rewritten in independent form, incorporating the substance of Claims 1 and 5. Claims 8-17 have been amended to only depend on allowable Claim 7. Further, new Claims 29-31 all depend on allowable Claim 7. Claim 6 has been rewritten in independent form, incorporating the substance of Claims 1 and 5.

Applicants respectfully submit new Claims 19-31 for examination, which all depend on originally filed Claim 6. No new matter has been added by way of the aforementioned Claim additions. For example, Applicant directs the Examiner's attention to originally filed Claims 1-17. Applicant submits that the identified sections are presented only for the Examiner's convenience and is not intended to be an exhaustive list of support.

Claims 1, 2, 4-6 and 18 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Tsugita, Akira et. al., "Additional Possible tools for identification of proteins on one or two dimensional electrophoresis", 1998, Electrophoresis, Vol. 19, pages 928-938 (hereinafter "Tsugita") in view of Covey et. al. U.S Patent No. 5,952,653 (hereinafter "Covey") and Xu, Naxing et. al., "Structural characterization of peptidoglycan muropeptides by matrix-assisted

laser desorption ionization mass spectrometry and postsource decay analysis," 1997, Analytical Biochemistry, Vol. 248, page 7-14 (hereinafter "Xu").

Claim 3 stands rejected under 35 U.S.C. 103(a) as being unpatentable over Tsugita in view of Covey and Xu, as applied to Claims 1-2, 4-6 and 18 above, and further in view of Harris, William A., et. al., "Use of matrix clusters and trypsin autolysis fragments as mass calibrants in matrix assisted laser desorption/ionization time-of-flight mass spectrometry," 2002, Rapid Communications in Mass Spectrometry, vol. 16, pages 1714-1722 (hereinafter "Harris").

Claims 8-17 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Tsugita in view of Covey and Xu.

In view of the following remarks, Applicants request further examination and reconsideration of the present patent application.

Rejections under 35 U.S.C. §103

Claims 1, 2, 4-6 and 18 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Tsugita in view of Covey and Xu. This rejection should be withdrawn based on the comments and remarks herein.

The combination of Tsugita, Covey and Xu does not render the claimed invention obvious for at least the following reasons. None of the references teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claims 1, 18 and all claims depending therefrom of the present application

Tsugita discloses a process for *C*-terminal sequencing for a protein, as disclosed in the Section "2.13 C-terminal sequencing", in which the C-terminal amino acid librated from the denatured protein (peptide) is modified with fluorescein isothiocynated and analyzed by HPLC, thereby the C-terminal sequence of the denatured protein (peptide) is analyzed without use of mass-spectroscopic analysis (See "2.13 C-terminal sequencing" of Tsugita)

The process for C-terminal stepwise degradation consists of the following three reaction sub-steps (i) - (iii):

(i) The first reaction sub-step for acetylation of the N-terminus of the peptide (denatured protein) and formation of an oxazolone at the C-terminal carboxyl group of the peptide (denatured protein): Acetic anhydride with 20% acetic acid tetrahydrofuran solution in the present of 1% DTT was reacted in liquid phase on the dried sample of peptide (denatured protein) at **60 °C** for 10 min. Alternatively, acetic anhvdride containing 10% acetic acid (1% DTT) vapor was reacted at room temperature for 20 min for the protein charged on the minicolumn.

(ii) The second reaction sub-step for degradation of the oxazolone-ring to liberate the C-terminal amino acid and to form the esterified peptide: The liquid phase reaction for degradation of the oxazolone-ring is made with 5% PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) in methanol (CH_3OH) at **5 °C** for 15 min.

(iii)The final reaction sub-step for conversion of the esterified peptide (peptidyl methyl ester) into the peptide with a free carboxyl group at its C-terminus: 10 % DMAE aqueous solution as used at **60 °C** for 20 min in the hydrolysis reaction of the ester bond to convert the esterified peptide (peptidyl methyl ester) into the peptide with a free carboxyl group.

Tsugita also discloses a process for Asp-C cleavage, as disclosed in the Section "3.2 Aspartic acid carboxyl side (Asp-C) cleavage", in which protein sample is cleaved at the carboxyl side of the aspartyl peptide bond (Asp-C) by a vapor phase reaction using 0.2% PFPA or HFBA aqueous vapor.

Tsugita employed such a specified cleavage condition for the Asp-C cleavage reaction that a vapor phase reaction was made with a **vapor** generated from a 0.2 % PFPA aqueous solution containing 1 % w/v DTT at 90 °C for 4-16 h. Tsugita reported that the Asp-C cleavage activates the newly exposed C-terminal aspartic acid and is often accompanied by the liberation of the aspartic residue at its C-terminus. Thus, the liberation of the aspartic residue at the C-terminus of the peptide fragment is induced by using **vapor** generated from a 0.2 % PFPA aqueous solution containing I % w/v DTI at 90°C. In such a case, formation of an oxazolone at the C-terminal Asp residue and degradation of the oxazolone-ring to liberate the C-terminal aspartic acid are by no means involved in the process for liberation of the aspartic residue by using **vapor** generated from a 0.2 % PFPA aqueous solution containing 1 % w/v DTT at 90°C. Tsugita also discloses a process for Ser/Thr-N cleavage, as disclosed in the Section "3.3 Amino side of serine/threonine (Ser-N) cleavage", in which protein sample is cleaved at the amino side of the serine/threonine peptide bond (Ser/Thr-N) by a vapor phase reaction **using PFPA or HFBA aqueous vapor**.

Tsugita, reported that the Ser/Thr-N cleavage will be made by such a vapor-phase reaction using **vapor** generated from a *75%* PFPA of HFBA aqueous solution at 50 °C for 24 hours. The reaction conditions often cause cleavages of acid-labile bonds such as Asp-Pro, Asp-C and glycine peptide bonds.

Tsugita also discloses a process for acid truncation of protein-multiple C-terminal sequencing from multi-sites, as disclosed in the Section "3.4 C-terminal sequencing at multiple sites", in which the C-terminal truncation reactions with the vapor of concentrated perfluoric acid, 90% PFPA at 90 °C, are accompanied by internal peptide cleavages occurring mainly at peptide bonds of Asp-C and Ser/Thr-N. The C-terminal truncation reactions also occur for the peptide fragments resulted from the internal peptide cleavages, and thus, C-terminal sequencing at multiple sites due to the internal peptide cleavages is made based on multiple sets of the series of C-terminal truncated peptide fragments without any further digesting the peptide fragments with trypsin.

Tsugita fails to provide any suggestion as to a suitable procedure for identifying a set of the series of C-terminal truncated peptide fragments that is produced from the C-terminal sequences of the parent protein from the numbers of the peptide fragments that are produced in the process for acid truncation of protein-multiple C-terminal sequencing from multi-sites.

The peptide fragment produced by Asp-C cleavage has Asp residue at its C-terminus. Thus, in the case if the peptide fragment produced by Asp-C cleavage is digested with trypsin, such a tryptic fragment having no Arg or Lys residue at its C-terminus is produced. The peptide fragment produced by Ser/Thr-N cleavage very often has other amino acid than Arg or Lys Thus, in the case if the peptide fragment produced by Ser/Thr-N cleavage is digested with trypsin, such a tryptic fragment having no Arg or Lys residues at its C-terminus is very often produced.

The tryptic fragments having no Arg or Lys residue at its C-terminus that are produced from the peptide fragments produced by the internal peptide cleavages will be hardly distinguished from the carboxyl terminus tryptic fragment that is produced from the C-terminal

portion of the parent protein, by using the **criteria based on the "double charge rule of tryptic fraqments having only one Arq or Lys residue at its C-terminus" as suggested in Covey**.

The tryptic fragments having no Arg or Lys residue at its C-terminus that are produced from the peptide fragments produced by the internal peptide cleavages will be hardly distinguished from the carboxyl terminus tryptic fragment that is produced from the C-terminal portion of the parent protein, by using the criteria for separating the tryptic fragments having only one Arg at its C-terminus from the tryptic fragments having no Arg or Lys at its C-terminus.

Accordingly, in the process for releasing the C-terminal amino acids successively, as claimed in currently amended Claim 1, the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages.

In contrast, Tsugita et al. fails to provide any suggestion as to suitable reaction conditions to be used for such process in which the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature, **but the reactions of releasing the C-terminal amino acids successively are by no means accompanied by any internal peptide cleavages.**

At the least, Tsugita fails to provide any suggestion about how to prevent internal peptide cleavages such as Asp-C cleavage and Ser/Thr-N cleavage in the case if vapor of perfluoric acid such as PFPA is used in combination with vapor of acetic acid anhydride. Indeed, Tsugita successfully uses vapor of perfluoric acid such as PFPA to induce internal peptide cleavages such as Asp-C cleavage and Ser/Thr-N cleavage at considerably high efficiency.

Further, Tsugita discloses formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring structure being carried out at different temperatures with the temperatures fluctuating. The protein of Tsugita is first undergoes chemical deblocking of the N-terminus at **-5°C**, followed by Asp-C cleavage at **90°C**, followed by Ser/Thr-N cleavage at either **30°C or 50°C**, followed by acid truncation at **90°C**, followed by C-teminal sequencing which includes reactions at **60°C**, **5°C** and again back to **60°C**. To summarize, the protein of Tsugita undergoes the following temperature fluctuation during formation and cleavage of the 5-oxazolone ring: **-5°C**, up to **90°C**, down to either **30°C or 50°C**, back up to **90°C**, down to **60°C**, down to **5°C** and back up again to **60°C**. This is a very large fluctuation in reaction temperatures and is representative of a temperature change of about **415°C** from beginning to end.

This is in contrast to the present application, in which that the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel and performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Covey et al. discloses a procedure of enzymatic digestion of the long peptide by trypsin to cleave the long peptide into tryptic fragments. Covey uses such Ion spray process for **Ion Evaporation Mass Spectrometry**, wherein the ionization of the liquid containing tryptic fragments is carried out by ion evaporation. In ion evaporation, the liquid to be ionized is dispersed into a large number of very small charged droplets. As the droplets evaporate and become smaller, the field strength in each droplet becomes sufficiently high that ions in the droplet are ejected intact from the droplet. Covey discloses such specific feature of the ion evaporation **that the locations of the charges on the gas phase ions produced by ion**

evaporation are at the same positions in the tryptic fragments as they are in the liquid solution phase. (See column 5 line 27 to line 42 of Covey)

Particularly, in the case when the tryptic fragments are dissolved in acidic aqua solution, for example, 0.5% formic acid used in Example 1 or 5%CH_3CN/0.1% trifiuoroacetic acid used in Example 2, the tryptic fragments in the acidic aqua solution phase are to be doubly charged because arginine or lysine at the C-terminus of each tryptic fragment and an amino terminus of each tryptic fragment are both positively ionized in the acidic aqua solution phase, whereas the carboxyi group at the C-terminus or carboxyl group of Asp or Glu are by no means negatively ionized.

In contrast, in the case if the tryptic fragments are dissolved in basic aqua solution of pH 10 or more, the tryptic fragments in the basic aqua solution phase will be negatively charged because arginine or lysine at the C-terminus of each tryptic fragment and an amino terminus of each tryptic fragment are by no means positively ionized in the acidic aqua solution phase, whereas the carboxyl group at the C-terminus or carboxyl group of Asp or Glu are both negatively ionized.

Indeed, it is well known that isoelectric focusing technique is based on such phenomenon that a protein or peptide that is in a pH region below its isoelectric point (pl) will be positively charged, whereas the protein or peptide that is in a pH region above its isoelectric point (pl) will be negatively charged.

As explained above, Covey only teaches such a double charge rule that, in the case when the tryptic fragments are dissolved in the acidic aqua solution, such as 0.5% formic acid or 0.1% trifluoroacetic acid aqua solution, the tryptic fragment having Arg or Lys at the C-terminus

thereof will be doubly positive-charged **in such form of $(M + 2H)^{2+}$ by the ion evaporation process**.

Further, Covey describes that, in the case when the tryptic fragments having Arg or Lys at their C-terminus and an amino group at their N-terminus are dissolved in the acidic aqua solution, normally between 90 and 100 percent of the tryptic fragment ions in the gas phase are doubly positive-charged (in such form of $(M + 2H)^{2+}$) when ion evaporation is used to convert them from the liquid to the gas phase. Thus, Covey clearly suggests that, in the case when the tryptic fragments having Arg or Lys at their C-terminus and an amino group at their N-terminus are dissolved in the acidic aqua solution, no more than 10 percent of the tryptic fragment ions in the gas phase are singly positive-charged (in such form of $(M+H)^{+}$) when ion evaporation is used to convert them from the liquid to the gas phase. In addition, Covey clearly suggests that, in the case when the tryptic fragments having Arg or Lys at their C-terminus and an amino group at their N-terminus are dissolved in the acidic aqua solution, no more than 10 percent of the tryptic fragment ions in the gas phase are singly negative-charged (in such form of $(M-H)^{-}$) when ion evaporation is used to convert them from the liquid to the gas phase.

Covey clearly suggests the first exception to the double charge rule that, in the case when a carboxyl terminus tryptic fragment which contains no Arg nor Lys but contains an amino group at its N-terminus is dissolved in the acidic aqua solution, the carboxyl terminus tryptic fragment ion will be only singly positive charged (in such form of $(M+H)^{+}$) when ion evaporation is used to convert it from the liquid to the gas phase. Covey clearly suggests that since a singly positive-charged fragment is far less frequent than the doubly positive-charged fragments in the case of the tryptic fragments having Arg or Lys at their C-terminus and an amino group at their N-terminus dissolved in the acidic aqua solution, therefore, singly positive-

charged ions observed by Ion Evaporation Mass Spectrometry tend to identify the tryptic fragment of the carboxyl terminus.

However, Covey fails to provide any suggestion as to how many percent of the carboxyl terminus tryptic fragment ions in the gas phase will be singly negative-charged (in such form of (M-H)') when ion evaporation is used to convert them from the liquid to the gas phase, in the case when the carboxyl terminus tryptic fragment is dissolved in the acidic aqua solution.

Covey also suggests the second exception to the double charge rule that, in the case when an amino terminus tryptic fragment which contains Arg or Lys at its C-terminus and contains a carboxylated or blocked amino group at its N-terminus (e.g. N-acylation at the N-terminus) is dissolved in the acidic aqua solution, the amino terminus tryptic fragment will be only singly positive charged (in such form of $(M+H)^+$) when ion evaporation is used to convert it from the liquid to the gas phase.

However, Covey, fails to provide any suggestion as to how many percent of the N-terminally carboxylated or blocked tryptic fragment ions in the gas phase will be singly negative charged (in such form of $(M-H)^-$) when ion evaporation is used to convert them from the liquid to the gas phase, in the case when the N-terminally carboxylated or blocked tryptic fragment is dissolved in the acidic aqua solution.

Further, Covey suggests the third exception to the double charge rule that, in the case when a tryptic fragment which contains Arg or Lys at its C-terminus, an amino group at its N-terminus, and an internal His is dissolved in the acidic aqua solution, a small percent of the tryptic fragment ions in the gas phase will be triply positive-charged (in such form of $(M + 3H)^{3+}$) and the remainder of the tryptic fragment ions in the gas phase will be doubly positive charged (in such form of $(M + 2H)^{2+}$) when ion evaporation is used to convert them from the

liquid to the gas phase. Therefore, Covey et al. clearly suggests that, in the case when a tryptic fragment which contains Arg or Lys at its C-terminus, an amino group at its N-terminus, and an internal His is dissolved in the acidic aqua solution, no more than 10 percent of the tryptic fragment ions in the gas phase may be singly positive-charged (in such form of $(M+H)^+$) when ion evaporation is used to convert them from the liquid to the gas phase. In addition, Covey clearly suggests that, in the case when a tryptic fragment which contains Arg or Lys at its C-terminus, an amino group at its N-terminus, and an internal His is dissolved in the acidic aqua solution, no more than 10 percent of the tryptic fragment ions in the gas phase may be singly negative-charged (in such form of $(M-H)^-$) when ion evaporation is used to convert them from the liquid to the gas phase.

Accordingly, Covey clearly suggests that, in the case when all of the tryptic fragments are dissolved in the acidic aqua solution, when ion evaporation is used to convert them from the liquid to the gas phase **for ion evaporation mass spectrometry of the tryptic fragments in positive mode**, the intensities of the singly positive-charged ions (in such form of $(M+H)^+$) from the tryptic fragments which contain Arg or Lys at its C-terminus and an amino group at its N-terminus, and from the tryptic fragment which contains Arg or Lys at its C-terminus, an amino group at its N-terminus, and an internal His will be much weaker that the intensities of the singly positive-charged ions (in such form of $(M+H)^+$) from the carboxyl terminus tryptic fragment which contains no Arg nor Lys but contains an amino group at its N-terminus, and from amino terminus tryptic fragment which contains Arg or Lys at its C-terminus and contains a carboxylated or blocked amino group at its N-terminus (e.g. N-acylation at the N-terminus).

However, Covey fails to provide any suggestion as to how many intensities of the singly negative-charged ions (in such form of $(M-H)^-$) from the four types of tryptic fragments will be

measured, in the case when all of the tryptic fragments are dissolved in the acidic aqua solution, when ion evaporation is used to convert them from the liquid to the gas phase for ion evaporation mass spectrometry of the tryptic fragments in negative mode.

Covey only suggests that, in the case when the tryptic fragments are dissolved in the acidic aqua solution, when ion evaporation is used to convert them from the liquid to the gas phase for ion evaporation mass spectrometry of the tryptic fragments in positive mode, the singly positive-charged ion from the tryptic fragment of the carboxyl terminus or the singly positive-charged ion form the tryptic fragment having an carboxylated or blocked amino terminus will be identified based on comparison of the intensity of the singly positive-charged ions (in such form of $(M+H)^+$) with the intensity of the doubly positive-charged ions (in such form of $(M + 2H)^{2+}$) .

However, Covey fails to provide any suggestion as to analytical procedure for identifying the singly positive-charged ion and singly negative-charged ion from the tryptic fragment of the carboxyl terminus based on comparison of the intensity of the singly positive-charged ions (in such form of $(M+H)^+$) with the intensity of the singly negative-charged ions (in such form of $(M-H)^-$), in the case when all of the tryptic fragments are dissolved in the acidic aqua solution, when ion evaporation is used to convert them from the liquid to the gas phase **for ion evaporation mass spectrometry of the tryptic fragments in positive mode and in negative mode**.

In addition, Covey fails to provide any suggestion as to whether or not such a double charge rule with three exceptions would be similarly observed in the case of MALDI-TOF-MS measurement in positive mode.

Indeed, MALDI-TOF-MS measurement employs Matrix assisted laser desorption/ionization (MALDI) process, whereas ion evaporation mass spectrometry employs the Ion evaporation process as explained above.

In the MALDI process, the matrix solution is mixed with the analyte (e.g. protein sample). This mixed solution is spotted onto a MALDI plate (usually a metal plate designed for this purpose). The solvents contained therein vaporize, leaving only the recrystallized matrix, but now with analyte molecules (protein) spread throughout the crystals. The matrix and the analyte are said to be co-crystallized in a MALDI spot.

The laser is fired at the crystals in the MALDI spot. The matrix absorbs the laser energy and it is thought that primarily the matrix is ionized by this event. The matrix is then thought to transfer part of its charge to the analyte molecules (e.g. protein), thus ionizing them while still protecting them from the disruptive energy of the laser. Ions observed after this process consist of a neutral molecule [MI and an added or removed ion. Together, they form a quasimolecular ion, for example $[M+H]^+$ in the case of an added proton, $[M+Na]^+$ in the case of an added sodium ion, or $[M-H]^-$ in the case of a removed proton.

As explained above, in the MALDI spot, the analyte (e.g. protein-sample) is present in the form of neutral molecule [M]. The ionization is caused by adding ion (H^+) from the photo-excited matrix or removing ion (H*) to the photo-excited matrix.

Accordingly, such a double charge rule with three exceptions that is a specific feature of the ion evaporation process in the case when the tryptic fragments are dissolved in the acidic aqua solution will be by no means observed in the MALDI process using dried-up matrix system.

At the least, Covey fails to provide any suggestion as to intensity of a singly positive-charged ion of (M+H)* from the tryptic fragment having Arg or Lys at the C-terminus thereof to be measured by MALDI-TOF-MS. Covey fails to provide any suggestion as to intensity of a singly negative charged ion of $(M-H)^-$ from the tryptic fragment having other amino acid than Arg or Lys at the C-terminus thereof to be measured by MALDI-TOF-MS.

At the least, Covey fails to provide such a suggestion that a singly positive-charged ion of $(M+H)^+$ from the tryptic fragment having Arg or Lys at the C-terminus thereof may show stronger intensity in the spectrum of the cationic species of $(M+H)^+$ measured by MALDI-TOF-MS.

More detailed explanation as to the technique of Covey, is given as follows. At first, Covey indeed uses a typical process, which comprises the steps of:

1) digesting the target protein with trypsin to produce mixture of the tryptic fragments containing only one Arg or Lys at its C-terminus and the C-terminal fragment containing no Arg of Lys;

2) separating each of the fragments from the mixture of the fragments produced in the step 1) by using chromatographic separation procedure such as HPLC;

3) separately mass-analyzing each of the fragments, which is separated from each another in step 2), by using Ion Evaporation Mass Spectrometry, wherein each of the fragments is mass-analyzed at the different scan, in which such a liquid sample of each of the fragments solved in an acidic aqueous solvent is ionized by ion evaporation.

In the acidic aqueous solvent such as 0.5% formic acid or 0.1% trifluoroacetic acid aqueous solution, the tryptic fragments containing only one Arg or Lys at its C-terminus is solved in such a doubly ionized cationic form, in which the carboxyl groups are not ionized, the N-terminal amino group is ionized in the form of $-NH_3^+$, and the amino group of Lys at the C-terminus is ionized in the form of $-NH_3^+$ or the guanidino group ($-NH-C(NH_2)-NH$) of Lys at the C-terminus is ionized in the form of $-NH-C^+(NH_2)-NH_2$.

On the other hand, in the acidic aqueous solvent such as 0.5% formic acid or 0.1% trifluoroacetic acid aqueous solution, the C-terminal fragment containing no Arg of Lys is solved in such a simply ionized cationic form, in which the carboxyl groups are not ionized and the N-

terminal amino group is ionized in the form of $-NH_3^+$. Therefore, in the ionization process using ion evaporation, the doubly ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus that is solved in the acidic aqueous solvent will be converted into the doubly ionized cationic species of $(M + 2H)^{2+}$ in gas phase at such a high efficiency as 90-100%. The doubly ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus that is solved in the acidic aqueous solvent may be converted into the singly ionized cationic species of $(M+H)^+$ in gas phase at a low efficiency. However, the doubly ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus that is solved in the acidic aqueous solvent will not be converted into such a singly ionized aionic species of $(M-H)^-$ in gas phase by ion evaporation.

On the other hand, the singly ionized cation of the C-terminal fragments containing no Arg or Lys that is solved in the acidic aqueous solvent will be converted into the singly ionized cationic species of $(M+H)^+$ in gas phase at a high efficiency. However, the singly ionized cation of the C-terminal fragments containing no Arg or Lys that is solved in the acidic aqueous solvent may not be converted into the singly ionized anionic species of $(M-H)^-$ in gas phase by ion evaporation.

In conclusion, in the case when each of the tryptic fragments containing only one Arg or Lys at its C-terminus and the C-terminal fragment containing no Arg of Lys solved in the acidic aqueous solvent is applied to the ion Evaporation Mass Spectrometry, only the cationic species will be observed in the positive mode spectrum, whereas any anionic species will be rarely observed in the negative mode spectrum.

Further, each of the tryptic fragments containing only one Arg or Lys at its C-terminus is separated from the mixture of the fragments by using chromatographic separation procedure such

as HPLC, so that only one doubly ionized cationic species of $(M + 2H)^{2+}$ in gas phase that is converted from the doubly ionized cation of each of the tryptic fragments containing only one Arg or Lys at its C-terminus solved in the acidic aqueous solvent is observed at the position of m/z=(M+2)/2 in each scan by the ion Evaporation Mass Spectrometry, as shown in FIG. 4A, 4B and 4C.

In such a case if the tryptic fragment containing only one Arg or Lys at its C-terminus further contains His residue, the tryptic fragments containing only one Arg or Lys at its C-terminus and His residue may be solved in such a triply ionized cationic form at a considerable percentage as well as in the doubly ionized cationic form at a high percentage. In such a case, additional triply ionized cationic species of $(M + 3H)^{3+}$ in gas phase that is converted from the triply ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus and His residue solved in the acidic aqueous solvent is observed at the position of m/z=(M+3)/3 by the Ion Evaporation Mass Spectrometry, as shown in FIG. 5.

The typical process disclosed in Ref. 2: Covey further comprises such a second stage of mass analysis in the well known MS-CID-MS mode. In the MS-CID-MS mode, the doubly ionized cationic species of $(M + 2H)^{2+}$ in gas phase that is converted from the doubly ionized cation of each of the tryptic fragments containing only one Arg or Lys at its C-terminus solved in the acidic aqueous solvent is subjected to collision induced dissociation in order to produce daughter ions from the doubly ionized cationic species of $(M + 2H)^{2+}$. In the step of collision induced dissociation, the doubly ionized cationic species of $(M + 2H)^{2+}$ is dissociated into a couple of daughter ions, i.e. a singly ionized cationic species of $(M_N+H)^+$ that is N-terminal daughter ion containing the N-terminal amino group ionized in the form of $-NH3^+$,

and a singly ionized cationic species of $(M_N+H)^+$ that is C-terminal daughter ion containing only one Arg or Lys with $-NH-C^+(NH_2)-NH_2$, or $-NH_3^+$ at its C-terminus, wherein $M_N + M_C = M$.

Thus, in the case if the spectrum in the MS-CID-MS mode is measured for each of tryptic fragments containing only one Arg or Lys at its C-terminus, plurality of couples of daughter ions, such as Y11 and B1,..., Y1 and B11 as illustrated in FIG.7, are observed around the position of m/z=(M+2)/2 for the parent ion that is the doubly ionized cationic species of $(M + 2H)^{2+}$. Such a fact that some of the daughter ions are observed at positions of m/z larger than (M+2)/2 provides good evidence proving that the parent ion observed at the position of m/z=(M+2)/2 is indeed the doubly ionized cationic species of $(M + 2H)^{2+}$, whereas a couple of daughter ions observed at m/z= $(M_N + 1)$ and m/z= $(M_c + I)$ are a singly ionized cationic species of $(M_N+H)^+$ and a singly ionized cationic species of $(M_N+H)^+$, respectively. Such feature of the spectrum observed in the MS-CID-MS mode for each of tryptic fragments containing only one Arg or Lys at its C-terminus is well illustrated in FIG. 8.

Accordingly, the typical process disclosed in Covey employs the spectrum observed in the MS-CID-MS mode in other to prove that the parent ion, which is observed at the position of m/z=(M+2)/2 in the spectrum of each scan by the Ion Evaporation Mass Spectrometry, is indeed the doubly ionized cationic species of $(M + 2H)^{2+}$.

Further, each of couples of daughter ions, such as Y11 and B1, as illustrated in FIG.7, is assigned in the spectrum measured in the MS-CID-MS mode by using such a criteria that a couple of daughter ions are observed at m/z= $(M_N + 1)$ and m/z= $(M_c + 1)$, wherein $M_N + M_C = M$, whereas the parent ion that is the doubly ionized cationic species of $(M + 2H)^{2+}$ is observed at the center position of m/z=(M+2)/2, as illustrated in FIG. 8.

Covey by no means uses any spectrum of anionic species in assignment of each of the fragments that is separated from the mixture of the fragments produced in the step 1), as no anionic species is observed for each of the fragments solved in the acidic aqueous solvent by the Ion Evaporation Mass Spectrometry.

Further, Covey does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Further, Covey does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel and performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Xu discloses a procedure of structural characterization of muropeptides derived from peptidoglycan by means of the MALD-MS based method.

Xu teaches such a prompt fragment ion peak $[MGlcNAc+Na]^+$ that is associated to the main (parent) ion peak $[M+Na]^+$ of Muropeptides, which is caused by the neutral loss of GlcNAc from the main (parent) ion due to the postsource decay process in the positive-ion MALDI-MS measurement. Xu also teaches such a prompt fragment ion peak $[M-GlcNAc-H]^-$ that is associated to the main (parent) ion peak $[M-H]^-$ of Muropeptides, which is caused by the

neutral loss of GlcNAc from the main (parent) ion due to the postsource decay process in the negative-ion MALDI-MS measurement.

Xu teaches such a major fragment ion peak $[b_{n-1}+Na+OH]^+$ that is caused by the loss of the C-terminal amino acid residue from the main (parent) ion $[M+Na]^+$ of Muropeptides due to the postsource decay process in the positive-ion MALDI-PSD mass spectrometry measurement. Xu teaches such a minor fragment ion peak $[b_n+Na-H]^+$ that is caused by the loss of the C-terminal amino acid residue from the main (parent) ion $[M+Na]^+$ of Muropeptides due to the postsource decay process in the positive ion MALDI-MS measurement. However, for example, the major fragment ion peak $[b_{n-1}+Na+OH]^+$ at m/z=920 is shown in FIG. 3, whereas the corresponding minor fragment ion peak $[b_{n-1}+Na-H]^+$ at m/z=920 is not shown in FIG. 3. Thus, such criteria based on such combination of the major fragment ion peak $[b_{n-1}+Na+OH]$ and its corresponding minor fragment ion peak $[b_n+Na-H]^+$ is by no means suitably used for identifying the fragment ion at m/z=920 as the major fragment ion peak $[b_{n-1}+Na+OH]^+$.

In view of this example, Xu fails to suggest that such criteria based on such combination of the major fragment ion peak $[b_{n-1}+Na+OH]^+$ and its corresponding minor fragment ion peak $[b_n+Na-H]^+$ is commonly available to identify the major fragment ion peak $[b_{n-1}+Na+OH]^+$ in the positive-ion MALDI-PSD mass spectrum.

Xu fails to suggest that such criteria based on such combination of the main Ion peak $[M+Na]^+$ and its corresponding minor ion peak $[M+Na-H-OH]^+$ would be commonly available to identify the main ion peak $[M+Na]^+$ in the positive-ion MALDI-PSD mass spectrum.

In contrast, such criteria 5a-1 and 5b-1 based on such corresponding ion peak having a m/z smaller than the m/z of the major ion peak by the molecular weight of 18 (the loss of H_20) are commonly available to identify the main ion peak $[M+H]^+$ of the tryptic fragment in the

MALDI-MS measurement in positive mode and the main ion peak $[M+H]^+$ of the tryptic fragment in the MALDI-MS measurement in negative mode.

Further, such criteria 5a-2 and 5b-2 based on such corresponding ion peak having a m/z larger that the m/z of the major ion peak by the molecular weight of acyl group (e.g. excess of CH_3-CO-) are commonly available to identify the main ion peak $[M+H]^+$ of the tryptic fragment in the MALDI-MS measurement in positive mode and the main ion peak $[M-H]^-$ of the tryptic fragment in the MALDI-MS measurement in negative mode.

In contrast, the loss of 203 mass units is only used to identify the prompt fragment ion peak $[M\text{-GlcNAc+Na}]^+$, which is caused by the neutral loss of GlcNAc from the main (parent) ion due to the postsource decay process in the positive-ion MALDI-MS measurement, and to identify the prompt fragment ion peak $[M\text{-GlcNAc-H}]^-$, which is caused by the neutral loss of GlcNAc from the main (parent) ion due to the postsource decay process in the negative-ion MALDI-MS measurement.

At the least, Xu fails to suggest that such criteria based on the loss of 203 mass units would be suitably employed to identify the main (parent) ion peak $[M+Na]^+$ of Muropeptides in the MALDI-MS measurement in positive mode and the main (parent) ion peak [M-HI- of Muropeptides in the MALDI-MS measurement in negative mode.

Xu fails to suggest that such criteria based on the loss of 203 mass units and 18 mass units would be suitably employed to identify the main (parent) ion peak $[M+Na]^+$ of Muropeptides in the MALDI-MS measurement in positive mode and the main (parent) ion peak $[M-H]^-$ of Muropeptides in the MALDI-MS measurement in negative mode. In contrast, such criteria 5a-3 and 5b-3 based on combination of excess of acyl group (e.g. excess of CH3-CO-) and loss of 18 (H_2O) are commonly available to identify the main ion peak $[M+H]^+$ of the tryptic

fragment in the MALDI-MS measurement in positive mode and the main ion peak $[M-H]^-$ of the tryptic fragment in the MALDI-MS measurement in negative mode.

Accordingly, such criteria 5a-1 and 5b-1, 5a-2 and 5b-2 and 5a-3 and 5b-3 are used in quite different way from the criteria based on the loss of 203 mass units and the criteria based on the decrease of 18 mass units of Xu. In this view, such criteria 5a-1 and 5b-1, 5a-2 and 5b-2 and 5a-3 and 5b-3 are by no means similar to the teaching of Xu.

Xu discloses the MALDI-TOF-MS spectra measured for the muropeptides derived from peptidoglycan by using lysostaphin digestion such as the positive-ion linear MALDl mass spectra shown in Figure *2*, in which $[M+Na]^+$ ion peaks are the dominant species, but $[M+H]^+$ ion peaks are by no means measured at any detectable level.

In particular, in MALDI-PSD analysis of an unsubstituted monomer, Xu clearly states "In positive-ion mode analysis, the $[M+Na]^+$ ion at m/z 991 was selected as the precursor for an unsubstituted muropeptide monomer because of its high abundance relative to the protonated molecules $[M+H]^+$."

In view of this disclsosure, Xu fails to suggest any use of the protonated molecules $[M+H]^+$ for the positive-ion mode analysis.

Accordingly, Xu fails to provide any suggestion that the protonated molecules $[M+H]^+$ will be used for structural characterization of peptides in combination with the deprotonated molecules $[M-H]^-$.

In particular, Xu clearly suggests that the major peak in the negative-ion MALDl mass spectrum is the deprotonated molecule [M-H]-, allowing unambiguous molecular weight determinations of unknown samples. (See page 10, left column). Indeed, Xu used only the ion peaks $[M-H]^-$ measured in the negative-ion MALDl mass spectrum to determine

the lysotophin digest of muropeptide dimer I and muropeptide dimer II.

In view of these facts, **Xu fails to suggest that the $[M+Na]^+$ ion peaks measured in the positive-ion MALDl mass spectrum are used in combination with the ion peaks $[M-H]^-$ measured in the negative-ion MALDl mass spectrum to determine unambiguous molecular weight of unknown samples.**

Further, Xu does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Further, Xu does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel and performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Therefore, the combination of Tsugita, Covey and Xu does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Further, since neither Tsugita, Covey nor Xu disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel being performed without extreme temperature fluctuations, the combination of Tsugita, Covey and Xu would not teach this claim recitation. Therefore, the combination of Tsugita, Covey and Xu does not render the claims of the present application obvious because the combination does not teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel being performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Thus, the rejection of Claims 1, 2, 4-6 and 18 under 35 U.S.C. §103(a) as being unpatentable over Tsugita in view of Covey and Xu is overcome. Withdrawal of the rejection and allowance of Claims 1, 2, 4-6 and 18 is earnestly solicited.

Claim 3 stands rejected under 35 U.S.C. §103(a) as being unpatentable over Tsugita in view of Covey and Xu, as applied to Claims 1-2, 4-6 and 18 above, and further in view of Harris.

The deficiencies of the combination of Tsugita, Covey and Xu are discussed above. Harris does not cure these deficiencies.

Harris discloses a method of using the $[M+H]^+$ ions from trypsin autolysis fragments as mass calibrants in the positive-ion mode MALDI-TOF based analysis.

However, Harris fails to disclose any experimental evidence suggesting that $[M-H]^-$ ions from the trypsin autolysis fragments will be successfully used as mass calibrants in the negative-ion mode MALDI-TOF based analysis. Further, Harris fails to provide any experimental evidence suggesting that the $[M-H]^-$ ions from the trypsin autolysis fragments will be successfully measured in the negative-ion mode MALDI-TOF based analysis.

Further, Harris does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel and being performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Further, Harris does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claim 1, from which Claim 3 depends.

Therefore, the combination of Tsugita, Covey, Xu and Harris does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claim 1, from which Claim 3 depends.

Further, since neither Tsugita, Covey, Xu nor Harris disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel and being performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application, the combination of Tsugita, Covey, Xu and Harris would not teach this claim recitation. Therefore, the combination of Tsugita, Covey, Xu and Harris does not render the claims of the present application obvious

because the combination does not teach or the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring being carried out in parallel and being performed without extreme temperature fluctuations, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Thus, the rejection of Claim 3 under 35 U.S.C. §103(a) as being unpatentable over Tsugita in view of Covey, Xu and Harris is overcome. Withdrawal of the rejection and allowance of Claim 3 is earnestly solicited.

Claims 8-17 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Tsugita in view of Covey and Xu. The Official Action states that Claims 8-17 would be allowable if rewritten to depend only on Claim 7. Claims 8-17 have been amended to only depend on Claim 7, overcoming the rejection. Withdrawal of the rejection and allowance of Claims 8-17 is earnestly solicited.

New Claims 19-28 have been added and depend on Claim 6. Claims 19-28 are patentable over the combination of Tsugita, Covey and Xu for the reasons discussed above, specifically because the combination does not disclose, teach or suggest the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids, but the reactions of releasing the C-terminal amino acids successively are not accompanied by any internal peptide cleavages as recited in Claims 6, from which Claims 19-28 depend. Therefore, New Claims 19-28 are patentable, allowance of Claims 19-28 are earnestly solicited.

For at least the reasons set forth in the foregoing discussion, Applicants believe that the Application is now allowable, and respectfully request that the Examiner reconsider the rejection and allow the Application. Should the Examiner have any questions regarding this Amendment, or regarding the Application generally, the Examiner is invited to telephone the undersigned attorney.

Respectfully submitted,

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